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der Vetsuisse-Fakultät Universität Zürich

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# The role of erythropoietin in the development of pulmonary hypertension

Inaugural-Dissertation

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To Martin

## Table of contents

## Abbreviations

| | |
|---|---|
| EMSA | electrophoretic mobility shift assay |
| eNOS | endothelial nitric oxide synthase |
| Epo | erythropoietin |
| $F_iO_2$ | fraction of inspired oxygen |
| $f_R$ | respiratory frequency |
| HIF | hypoxia-inducible factor |
| NO | nitric oxide |
| PCR | polymerase chain reaction |
| PHD | prolyl-hydroxylase |
| rhEpo | recombinant human erythropoietin |
| RV $dP/dt_{max}$ | maximal rate of right ventricular pressure increase |
| RVSP | right ventricular systolic pressure |
| tg6 | transgenic mouse line overexpressing human Epo cDNA |
| $V_E$ | minute ventilation |
| $V_T$ | tidal volume |
| wt | wild-type mouse |

## 1. Summary

Besides enhancing erythropoiesis, erythropoietin (Epo) has several tissue-protective effects. We tested the impact of elevated Epo plasma levels on pulmonary hypertension and made use of the transgenic mouse line tg6 chronically overexpressing Epo and reaching haematocrit levels of up to 89%. Mice were exposed to normobaric hypoxia (≥ 7% $O_2$) for up to 24 h. Haemodynamic measurements showed a smaller increase in pulmonary artery pressure in hypoxic tg6 mice compared to wild type (wt) controls. Plethysmographic analysis and blood gas determination revealed impaired ventilation and reduced blood oxygenation. Hypoxic tg6 lung tissue showed a more pronounced stabilisation of HIF-1α and -2α as well as HIF binding activity. When comparing hypoxic lung tissue of tg6 and wt animals we did not observe altered mRNA expression of all three prolyl hydroxylases that are important modulators of HIF stabilisation. Interestingly, eNOS mRNA level was elevated in tg6 mice and the NO synthase inhibitor L-NAME reduced the elevation of HIF-1α and -2α protein levels in the lung of hypoxic tg6 mice to a higher extent than in wt. We also replaced tg6 by wt animals that were acutely treated with recombinant human Epo. As we did not observe any difference between Epo-treated and untreated wt mice, we conclude that the protective effects observed in hypoxic tg6 mice are not due to acute effects of Epo, but the result of adaptive mechanisms responding to Epo-induced chronic excessive erythrocytosis.

## 2. Zusammenfassung

Erythropoietin (Epo) hat, neben der Erythropoiese, verschiedene Gewebe-schützende Effekte. Wir haben den Einfluss von erhöhten Epo-Werten im Plasma auf die pulmonäre Hypertonie untersucht und das Modell der transgenen Maus (tg6) mit chronischer Überexpression von Epo und einem Hämatokrit von bis zu 89% verwendet. Die Mäuse wurden bis zu 24 h lang einer normobaren Hypoxie (≥ 7% $O_2$) ausgesetzt. Hämodynamische Messungen zeigten einen kleineren Anstieg des pulmonären Arteriendruckes in hypoxischen tg6 Mäusen im Vergleich zu den Kontrollen (wt). Plethysmographische Analysen und Blutgasbestimmungen ergaben eine beeinträchtigte Atmung und Sauerstoffsättigung des Blutes. Lungengewebe von hypoxischen tg6 Mäusen zeigte eine erhöhte HIF-1α und -2α Stabilisierung und HIF Bindungsaktivität. Wenn man hypoxisches Lungengewebe von tg6 und wt Tieren vergleicht, beobachtet man keine Veränderung in der mRNA Expression der drei Prolyl-hydroxylasen, den wichtigsten Modulatoren der HIF Stabilisierung. Interessanterweise war die eNOS mRNA in tg6 Mäusen erhöht, währenddem der NO Synthase Hemmer L-NAME den Anstieg von HIF-1α und HIF-2α Protein in der Lunge von hypoxischen tg6 Mäusen stärker reduzierte als in wt. Wir haben die tg6 mit wt Tieren ersetzt, die wir mit rekombinantem humanem Epo behandelt haben. Da wir keinen Unterschied zwischen Epo-behandelten und unbehandelten wt Mäusen beobachtet haben, folgern wir, dass der protektive Effekt von Epo in hypoxischen tg6 Mäusen kein akuter Effekt von Epo darstellt, sondern das Ergebnis von Anpassungsmechanismen aufgrund der Epo-induzierten chronischen exzessiven Erythrozytose.

## 3. Introduction

Chronic alveolar hypoxia occurring at high altitude (above 2500m) or in chronic respiratory or cardiorespiratory diseases such as emphysema, chronic bronchitis or cystic fibrosis might lead to pulmonary hypertension (Wagner 2001; León-Velarde 2008). This is due to the fact that decreased levels of alveolar oxygen concentrations can cause vasoconstriction in the pulmonary circulation. During prolonged hypoxia, an imbalance in the release of vasoactive substances and an increase in muscularisation in the small pulmonary arteries (media hypertrophy) might occur, leading to an increased pulmonary vascular resistance and sustained elevation of pulmonary arterial pressure (Arias Stella 1963; Meyrick 1980; Reid 1986; Lüscher 1990; Matsuoka 2001). With time the resulting pulmonary hypertension will lead to right ventricular hypertrophy (Peñaloza 1971; Meyrick 2001; Weissmann 2001). As a consequence, impaired blood oxygenation and tissue hypoxia will occur. The exact mechanisms of pulmonary hypertension are not fully understood, but a variety of factors, including hypoxia, erythropoietin (Epo), vasoactive substances (particularly nitric oxide), pulmonary vascular remodelling, and altered reactivity of the pulmonary vasculature have been implicated in the disease process. In the present study we investigated the influence of high Epo plasma levels in the development of hypoxia-induced pulmonary hypertension.

Epo is a pleiotropic cytokine that is upregulated during prolonged alveolar hypoxia and enhances erythropoiesis (Eckardt 2005; Sasaki 2003; Fandrey 2004; Stockmann 2006). Binding of Epo to its receptor on the erythrocyte progenitor cells present in the bone marrow maintains the viability of the cells, promotes cell division, and increases the haemoglobin synthesis followed by increased haematocrit levels (Fisher 2003; Jelkmann 2005). Epo therefore improves the oxygen carrying capacity of the blood, which in turn supports coping with low inspired oxygen concentrations. Interestingly, Epo also has several non-erythropoietic functions (Gassmann 2003). Many recent studies have shown that Epo has a protective function in the heart (Brines 2004; Parsa 2004; Shi 2004; Joyeux-Faure 2005; Joyeux-Faure 2007; Bahlmann 2008) and in several neural stroke models (Gassmann 2003; Brines 2004; Ghezzi 2004; Marti 2004). A neuroprotective role of Epo in light-induced retinal degeneration (Grimm 2002; Grimm 2004; Kilic 2005) and mechanical and ischemic injury of the

spinal cord (Gorio 2002; Celik 2002) has also been reported. Moreover, we have recently demonstrated that Epo expressed in the brain also affects the neural cardiorespiratory network by increasing hypoxic minute ventilation (Soliz 2005; Soliz 2007b) and by doing so improves oxygenation during hypoxia. Unfortunately, despite the beneficial effects, excessive erythrocytosis caused by Epo also has a pathological impact. The increased haematocrit might lead to high blood viscosity and therefore to a higher risk of cardiovascular disorders such as life-threatening cyanosis, hyperaemia, pulmonary hypertension and a high risk of thrombosis (Monge 1976; Vogel 2003; Reeves 2004; Heinicke 2006).

To further investigate the role of Epo and excessive erythrocytosis in the development of pulmonary hypertension, we used a transgenic mouse line (tg6 mouse). These mice develop chronic excessive erythrocytosis due to a constitutive overexpression of human Epo (Ruschitzka 2000; Wagner 2001; Heinicke 2006; Soliz 2007a) that results in haematocrit values of up to 0.89. This leads to an increased blood volume (Ruschitzka 2000; Wagner 2001; Shibata 2003; Vogel 2003) and elevated central venous and pulmonary artery pressure (Ruschitzka 2000; Wagner 2001). Surprisingly, tg6 mice do not show an increase in arterial blood pressure, no right ventricular hypertrophy and no increased muscularisation of the pulmonary vasculature. They have a normal blood pressure, heart rate and cardiac output (Ruschitzka 2000; Wagner 2001; Vogel 2003). Furthermore, there is no increased risk of thrombosis (Wiessner 2001; Shibata 2003). The excessive erythrocytosis in tg6 mice therefore leads to an elevated pulmonary artery pressure but not to other problems found in animals with hypoxia-induced pulmonary hypertension. We postulate that this is due to an adaptation to the high haematocrit levels as a result of the slow development of erythrocytosis over the first weeks during the animal's growth (Wagner 2001). Tg6 mice show increased expression of endothelial nitric oxide synthase (eNOS) in the endothelial cells of the pulmonary arteries, thereby leading to elevated synthesis of nitric oxide (NO) (Ruschitzka 2000; Hasegawa 2004). This could be due to the increased Epo levels, as others have reported that Epo could have a direct activating effect on NO synthase in endothelial cells (Yamane 1999; Banarjee 2000). It has been shown, that tg6 survival critically depends on NO bioavailability (Ruschitzka 2000; Hasegawa 2004). In turn, NO induces vasorelaxation and inhibits platelet activation (Ruschitzka 2000) despite the

increased endothelin-1 levels in endothelial cells of tg6 mice (Quaschning 2003). Apart from the NO-mediated vasodilatation, it was demonstrated that adaptation to excessive erythrocytosis in tg6 mice also includes regulation of blood viscosity by improving the red blood cell deformability and most probably aggregation (Starzyk 1999; Vogel 2003; Vogel 2004). Furthermore, the portion of large vessels is increased whereas the portion of small vessels is decreased. Also, the vessels have reduced pulmonary vascular smooth muscle thickness (Weissmann 2005). In this way, the transgenic animals manage to reduce vascular tone and responsiveness. Since Epo, high haematocrit levels, NO and endothelin-1 are believed to be involved in the effects of hypoxia in the lung (Grimminger 1995; Durmowicz 1999), structural and functional adaptations to hypoxia in the pulmonary circulation are probably altered in tg6 mice compared to wt mice, with tg6 mice exhibiting anti-pulmonary hypertensive effects (Weissmann 2005). Since in chronic pulmonary disease both, hypoxemia and polycythaemia are simultaneously present, their individual effects on the development of pulmonary hypertension are difficult to define. The tg6 mouse model allows us to distinguish between direct effects of hypoxia and secondary hypoxic effects due to the excessive erythrocytosis. To distinguish between direct effects of Epo and effects due to the increased haematocrit that follows high Epo plasma levels, we compared tg6 mice to wild-type (wt) mice and wt mice treated with recombinant human Epo (rhEpo) prior to the experiments.

Epo synthesis induced by low oxygen supply to the tissue is regulated by transcription factors hypoxia-inducible factor-1 and 2 alpha (HIF-1α and HIF-2α) that bind to the HIF binding site on the Epo gene and thereby induce the transcription of Epo (Jewell 2001). HIF-α subunits are constitutively expressed but under normoxic conditions they are quickly degraded by the ubiquitin-proteasome pathway. The proteosomal degradation of HIF-α subunits is induced by a $Fe^{2+}$-dependent hydroxylation of specific prolyl residues within the oxygen-dependent degradation domain of HIF-α subunits caused by prolyl-hydroxylases (PHDs). The activity of the PHDs depends on the availability of oxygen and is decreased under hypoxic conditions (Epstein 2001; Stroka 2001). PHDs therefore appear as today's best candidates for cellular HIF oxygen sensors (Fandrey 2006). Under hypoxic conditions, HIF-α subunits are stabilised and heterodimerize with the constitutively expressed HIF-1β subunits and form the HIF complexes, which move into the

nucleus and bind to the HIF-binding sites of HIF target genes (Semenza 2002; Wenger 2002). More than 70 HIF target genes are known (Wenger 2005), all of them stimulating either the anaerobic energy production via glycolysis or improving the tissue oxygenation by stimulating angiogenesis, vasodilatation or erythropoiesis and ultimately help to cope with hypoxia.

To evaluate the impact of Epo and excessive erythrocytosis on the development of hypoxia-induced pulmonary hypertension we performed haemodynamic measurements, measuring the right ventricular systolic pressure (RVSP) under normoxic and hypoxic conditions in tg6 mice, wt mice and wt mice treated with rhEpo prior to the measurements. As expected, we recorded a higher RVSP in tg6 mice compared to wt mice under normoxic conditions. Because RVSP influences the gas exchange in the lung, blood gas analysis showed that tg6 mice have an impaired gas exchange or ventilation. To study this phenomenon, we also analysed the ventilation of tg6 mice compared to wt mice. To study the impact of decreased blood oxygenation of tg6 mice on lung tissue, we measured mRNA and protein levels of HIF, the HIF binding activity, the levels of PHD1, 2 and 3 mRNA and also eNOS mRNA in lung tissue.
The data obtained in the present study suggest that excessive erythrocytosis, which represents a secondary effect of Epo, influences the development of pulmonary hypertension, but not Epo itself.

## 4. Materials and Methods

All animal experiments were performed in accordance with Swiss animal protection laws and Zurich University institutional guidelines and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

### 4.1 Transgenic mice

The transgenic mouse line was generated by pronuclear injection of the full-length human Epo cDNA driven by the human platelet-derived growth factor (PDGF) B-chain promoter and has been described previously (Ruschitzka 2000). The resulting mouse line TgN(PDGFBEPO)321ZbZ (tg6) was bred by mating hemizygous males to wild-type (wt) C57BL/6 females. Half of the offspring was hemizygous for the transgene, and the other half was wt and served as control. For the experiments we took only female mice, and they were used at three month of age.

### 4.2 Haemodynamic measurements

For the haemodynamic measurements, the animals were anaesthetised by 1.5% isoflurane and a catheter was placed in the right femoral vein. Thereafter, anaesthesia was maintained by continuous infusion of etomidate (10 mg/kg/h). Right ventricular pressure was measured in closed-chest spontaneously breathing mice as previously described (Deten 2004). Briefly, an ultra miniature catheter pressure transducer (2F model SPR-612, Millar Instruments Inc., Houston, TX, USA) was inserted into the right jugular vein and advanced into the RV via the right atrium. Room air was supplied via a nose mask. After a short stabilisation period of about 10 min, hypoxia was generated through mixing nitrogen and inspiratory air by a pump (DIGAMIX 6KM301, Wösthoff Messtechnik GmbH, Germany), starting with a reduction of the fraction of inspired $O_2$ ($F_iO_2$) to 18%. Thereafter, inspiratory oxygen concentration was further reduced by 2% every 7 min and RV function was continuously recorded.

### 4.3 Blood gas analyses

Blood gas was measured in animals that have been used for the haemodynamic measurements. 7 min after reaching the inspiratory oxygen concentration of 10% or 6%, the mice were euthanized. Arterial blood was drawn from a catheter placed in the right carotid artery and blood gas analysis was performed immediately on a bloody machine (Stat Profile, pHOx-Serie, Nova Biomedical, Labor Systeme Flükiger AG, Switzerland) according to manufacturer's instructions.

### 4.4 Ventilatory measurements

A whole-body flow-though plethysmograph (EMKA Technologies, France) was used to monitor ventilation as described (Soliz 2005). Briefly, mice were placed in a 600 ml chamber continuously supplied with airflow at 0.7-0.8 l $min^{-1}$ using flow restrictors. Ventilation ($V_E$) was calculated as the product of tidal volume ($V_T$) and respiratory frequency ($f_R$) and normalized to 100 g of body weight. Ventilatory measurements were performed in normoxia (21% $O_2$) and acute hypoxia achieved by flushing air balanced in $N_2$. The $F_iO_2$ in the chamber was gradually decreased from 21% to 10% $O_2$ during 15 min. Respiratory recordings at 10% $O_2$ were performed for 6 h.

### 4.5 Hypoxic exposure

Mice were exposed to hypoxia in an Invivo2 1000 hypoxic workstation (Ruskinn, UK). After placing the cages containing the animals in the workstation and a short adaptation period the oxygen concentration was gradually reduced by 2% every 10 min until the desired oxygen concentration was reached. At the end of the hypoxic exposure period mice were sacrificed by cervical dislocation. Thereafter, the thorax was quickly opened and the lung was frozen in liquid nitrogen for later mRNA and protein analyses. The duration of the tissue extraction procedure was about 30 – 40 s with little variation. In order to assure best comparability, each lobe of the lung was stored separately and corresponding whole lobes were used for each analysis, i.e. Western blot, EMSA, mRNA expression (see below).

### 4.6 Drug application

Recombinant human Epo (5.000 U/kg, s.c.) or L-NAME (50mg/kg, i.p.) was applied 30 min prior to hypoxic exposure.

### 4.7 Protein extraction

The tissue samples were homogenized in lysis buffer (10 mM Tris-HCl (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl mixed with protease inhibitors (Protease Inhibitor Cocktail (Calbiochem, USA), 1mM Vanadate, 0.5 MM PMSF, 0.5 mM DDT) with the Ultra-Turrax (IKA, Faust, Switzerland). Then the samples were homogenized in a glass douncer with a tight-fitting pestle for 30 strokes on ice and centrifuged at 1000 x g for 5 min. The nuclear pellets were resuspended in nuclear extraction buffer (20 mM Tris-HCl (pH 7.5), 1.5 mM $MgCl_2$, 420 mM KCl, 20% Glycerol mixed with protease inhibitors), rotated for 30 min at 4°C and then centrifuged for 30 min at 13.000 x g. The protein concentrations were measured with the BioRad Protein Assay.

### 4.8 Western blot analysis

Nuclear protein extracts (50 µg) were separated by denaturing SDS-page and transferred to a nitrocellulose membrane. After blocking with 5% non-fat dry milk in TBS, the membranes were incubated overnight at 4 °C with the primary antibody, HIF-1$\alpha$ and HIF-2$\alpha$ (both Novus biologicals, USA) at a dilution of 1:500 and 1:1000, respectively. The membranes were washed with TBS containing 0.05% Tween and incubated for 1 h at room temperature with the hrp-conjugated secondary antibody (Jackson Immuno Research Laboratories, USA, 1:5000). The membranes were then incubated with a luminol reaction solution (100mM Tris-HCl (pH 8.5), 0.1 mM coumaric acid, 0.625 mM luminol) and exposed to the Luminescent Image Analyzer LAS-3000 (Bucher Biotec AG, Switzerland). For normalisation, the blots were stripped in 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 0.7% β-Mercaptoethanol at 50 °C for 5 – 15 min and subsequently reprobed for SP-1 (Santa Cruz Biotechnology, Germany, 1:500). The digital images were quantified by Quantity One software (Bio-Rad Laboratories AG, Switzerland).

### 4.9 Electrophoretic mobility shift assay (EMSA)

Positive and negative strands of W18 and M18 oligonucleotide (Integrated DNA Technologies, USA), were annealed at 95 °C for 1 min. The double stranded W18 oligonucleotides were labelled by T4 polynucleotide kinase (Fermentas, Lab Force AG, Switzerland) using 50 µCi [γ-32P]-ATP. Unincorporated dsDNA were removed by filtration over DNA Quick Spin Column (Sephadex G-25, Roche Diagnostics, USA). Pre- and postcolumn samples were counted in a Liquid Scintillation Analyzer (Canberra Packard, Switzerland). For the EMSA, 5 µg nuclear protein extract was mixed with 0.4 - 0.7 ng 32P-labelled W18 probe, 1 µg M18 probe, and 75 ng Poly dI-dC in DNA binding buffer and incubated for 20 min at room temperature. Thereafter, a 5% non-denaturing polyacrylamide gel electrophoresis was performed at 300 V and 4 °C, the gel was dried and the probes were visualized using a Molecular Imager FX (Bio-Rad Laboratories AG, Switzerland). Specifity was verified by competition with unlabeled W18 oligonucleotides and supershift with HIF-1α antibody.

### 4.10 RNA extraction

Total RNA was isolated using the Trizol-Reagent (Invitrogen, USA) according to the protocol supplied by the manufacturer. RNA concentrations were determined spectrophotometrically, and RNA integrity was monitored by denaturing formaldehyde/agarose gel electrophoresis.

### 4.11 Quantitative real-time PCR

For quantitative real-time PCR, 5 µg total RNA was reversely transcribed using oligo(dT) and reverse transcriptase (Invitrogen, USA) according to the protocol supplied by the manufacturer. The mRNA levels of PHD1, PHD2 and PHD3 as well as of HIF-1α (forward and reverse primer TCAAGTCAGCAACGTGGAAG and CGGCTCATAACCCATCAACT, respectively) and HIF-2α (forward and reverse primer TGAAGCTGAGGCCGACCA and GCCGACTTGAGGTTGACAG, respectively) were quantified with 5 µl of diluted cDNA reaction (corresponding to 2.5% of cDNA reaction) using a SybrGreen qPCR reagent kit (SIGMA-ALDRICH, Switzerland) in combination with an ABI 7500 FAST light cycler (Applied Biosystems, Switzerland).

Initial template concentrations of each sample were calculated by comparison with serial dilutions of a calibrated standard. To control for equal input levels, ribosomal protein S12 mRNA was determined, and data were expressed as ratios relative to S12 levels. Melting point analyses and agarose gel electrophoresis of amplified PCR products were performed to quantify mRNA levels of eNOS by using 5 µl of diluted cDNA reaction (corresponding to 2.5% of cDNA reaction) and TaqMan Fast Universal PCR Master Mix in combination with an ABI 7500 FAST light cycler (all Applied Biosystems, Switzerland). Initial template concentrations of each sample were calculated by comparison with serial dilutions of a calibrated standard. To control for equal input levels, ribosomal protein S28 mRNA was determined, and data were expressed as ratios relative to S28 levels.

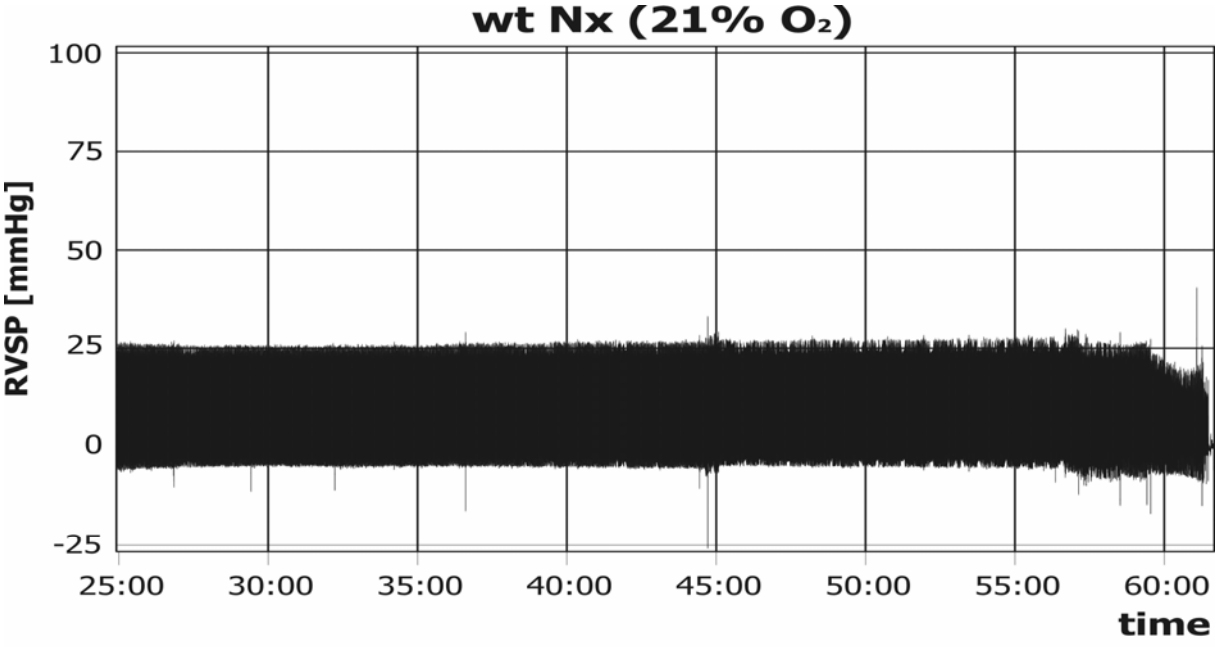
### 4.12 Statistical analyses

One way or two way ANOVA was used, subsequently utilizing the Hom-Sidak post hoc procedure (SigmaStat 3.5, Systat Software Inc.). A value of $p < 0.05$ was considered statistically significant.

# 5. Results

## 5.1 Haemodynamic measurements

Tg6 and wt mice were exposed to gradually decreasing inspiratory oxygen concentrations from 21% to 6% oxygen while right ventricular systolic pressure (RVSP) and maximal rate of right ventricular pressure increase (RV $dP/dt_{max}$) were continuously measured. To evaluate whether the measured differences in tg6 and wt mice were due to high Epo plasma levels or the resulting excessive erythrocytosis, we injected rhEpo in wt mice prior to the experiment. As expected, normoxic RVSP and RV $dP/dt_{max}$ were higher in tg6 mice compared to wt mice (Fig. 1A and Fig. 1B). Under hypoxic conditions, RVSP and RV $dP/dt_{max}$ increased dose-dependently in wt mice, and the dose-response curve to decreasing inspired oxygen peaked at 8% $O_2$ concentration. In tg6 mice, we did not observe a statistically relevant change in RVSP and RV $dP/dt_{max}$, but we measured the highest values at 12% inspired $O_2$. Furthermore, not all of the tg6 mice survived an inspired $O_2$ concentration of 6%. We did not see a difference in RVSP and RV $dP/dt_{max}$ in wt and Epo treated wt mice, neither in normoxia nor in hypoxia.


wt Nx (21% O₂)
RVSP [mmHg]
100
75
50
25
0
-25
25:00
30:00
35:00
40:00
45:00
50:00
55:00
60:00
time

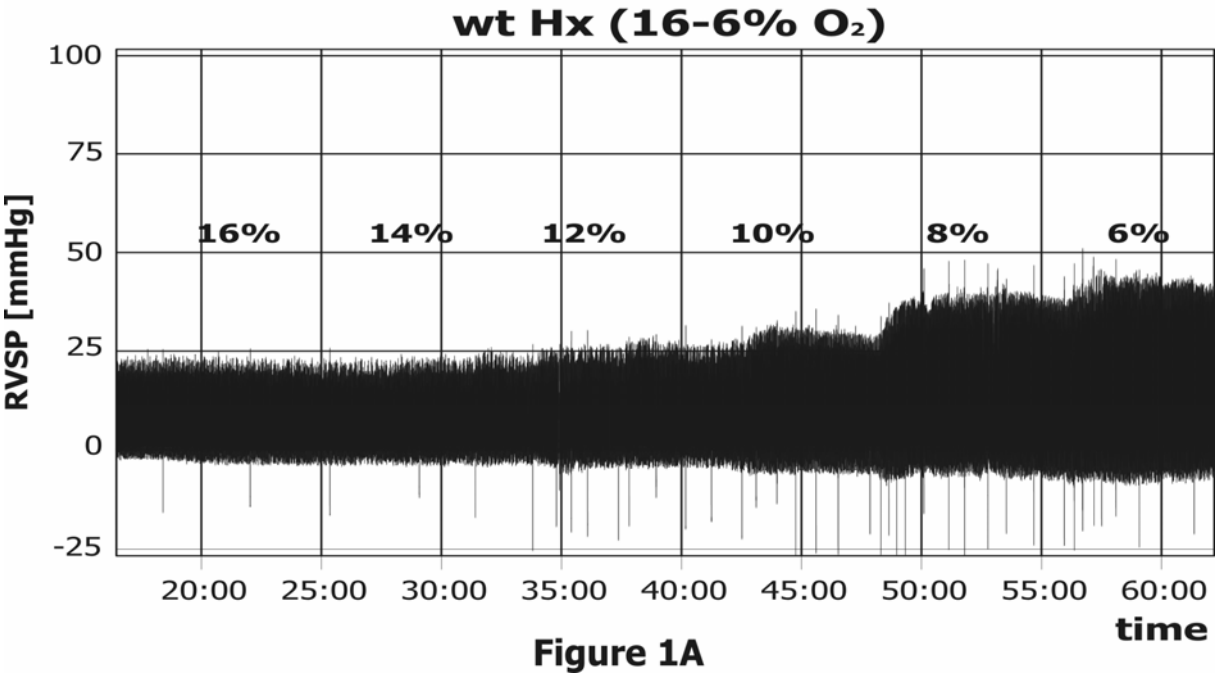

wt Hx (16-6% O₂)
RVSP [mmHg]
100
75
50
25
0
-25
16%
14%
12%
10%
8%
6%
20:00
25:00
30:00
35:00
40:00
45:00
50:00
55:00
60:00
time

**Figure 1A**

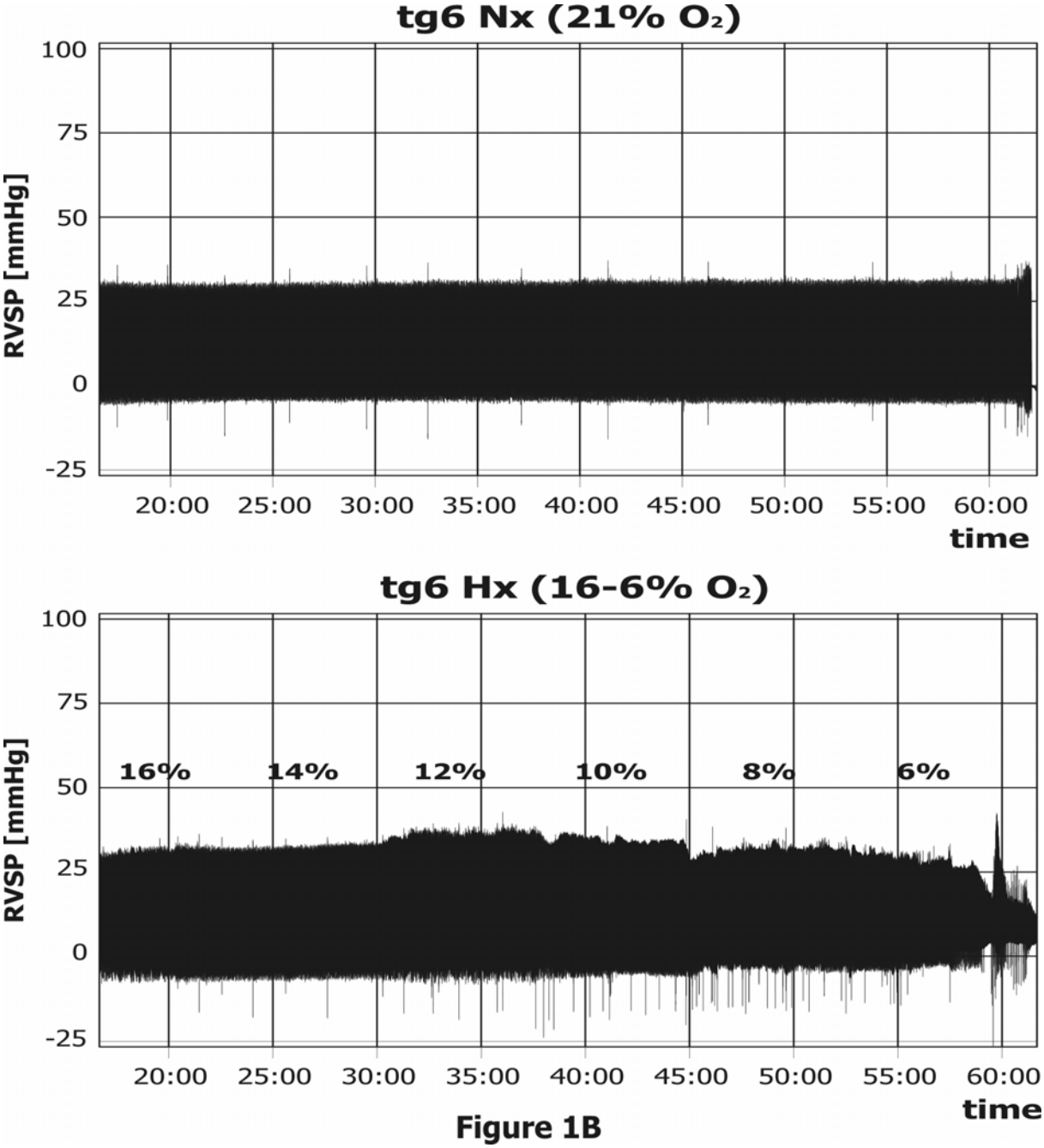

tg6 Nx (21% O₂)
RVSP [mmHg]
100
75
50
25
0
-25
20:00
25:00
30:00
35:00
40:00
45:00
50:00
55:00
60:00
time
tg6 Hx (16-6% O₂)
RVSP [mmHg]
16%
14%
12%
10%
8%
6%
time

Figure 1B

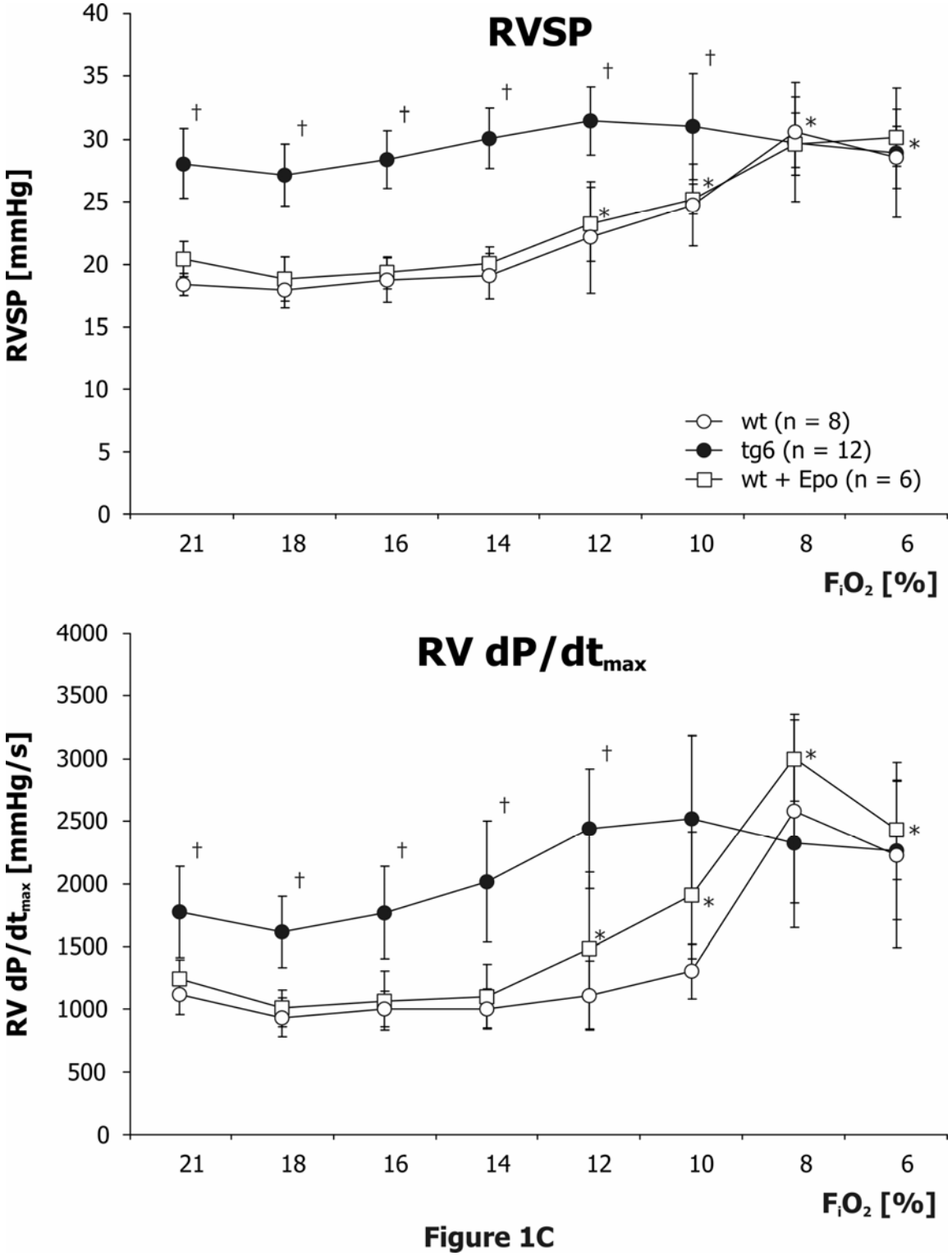



**Figure 1C**

**Figure 1**: Exemplary original recordings (Fig. 1A and Fig. 1B) and haemodynamic data of tg6 and wt mice during decreasing inspiratory oxygen concentrations as indicated (Fig. 1C). RVSP: right ventricular systolic pressure; RV $dP/dt_{max}$: maximal rate of right ventricular pressure increase. Numbers of animals at the beginning of the experiment are given in parentheses. Four of the tg6 and four of the wt mice were bled after exposure to 10% inspiratory $O_2$ for blood analyses and did not complete the experiment. Four additional of the tg6 mice, but none of the wt or Epo treated wt mice, did not complete the protocol (one died shortly after reduction of the inspiratory $O_2$ to 10%, one after 8% and two after 6%). Values in normoxic control mice (room air) did not change over time in both genotypes and are not shown in the summary. Data are mean ± SD. * $p < 0.05$ vs corresponding Nx; † $p < 0.05$ vs corresponding wt.

### 5.2 Blood gas analysis

Eight of the tg6 and eight of wt mice that were used for the haemodynamic measurements were euthanized after reaching 10% or 6% inspiratory $O_2$. Blood gas analysis was performed immediately after the blood was gained from the catheter placed in the right carotid artery. Normoxic animals served as controls. In hypoxia, we observed a decrease in $pO_2$, $SO_2$ and $pCO_2$ in both, tg6 and wt mice. Tg6 mice had a lower $pO_2$ and $SO_2$ but a higher $pCO_2$ compared to wt mice.

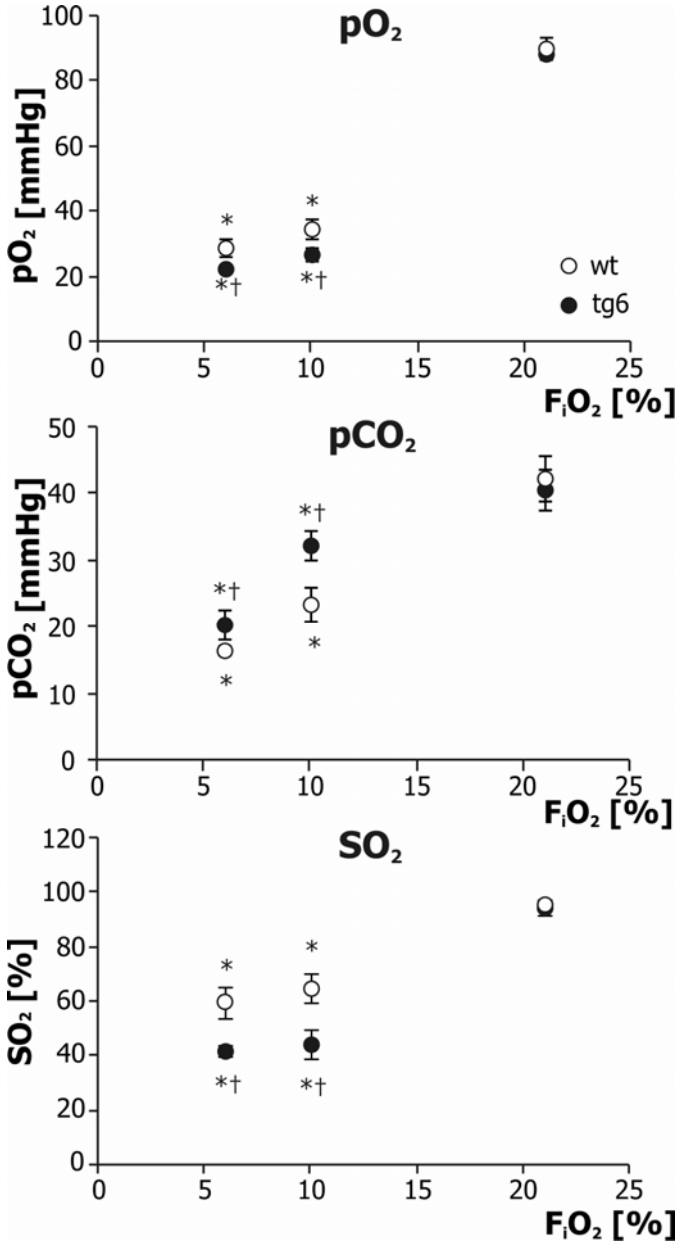



**Figure 2**: Blood gas analysis of tg6 and wt mice in normoxia and after 10% or 6% inspiratory oxygen concentration. For each group n = 4. Data are mean ± SD. * $p < 0.05$ vs corresponding Nx; † $p < 0.05$ vs corresponding wt.

### 5.3 Analysis of ventilation

Ventilation was measured in normoxia and after exposure to 10% inspiratory $O_2$ for 6 h by plethysmography. Respiratory frequency ($f_R$) and tidal volume ($V_T$) were determined and minute ventilation ($V_E$) was calculated as the product of $f_R$ and $V_T$ ($V_E = f_R * V_T$). We observed a temporary increase in minute ventilation that was most probably due to a temporary increase in breathing rate in wt and tg6 animals, but the increase in tg6 mice lasted much longer. After a short adaptation period, the $V_E$ of tg6 mice dropped below the level of wt mice. This could be due to the decrease in tidal volume in tg6 mice.

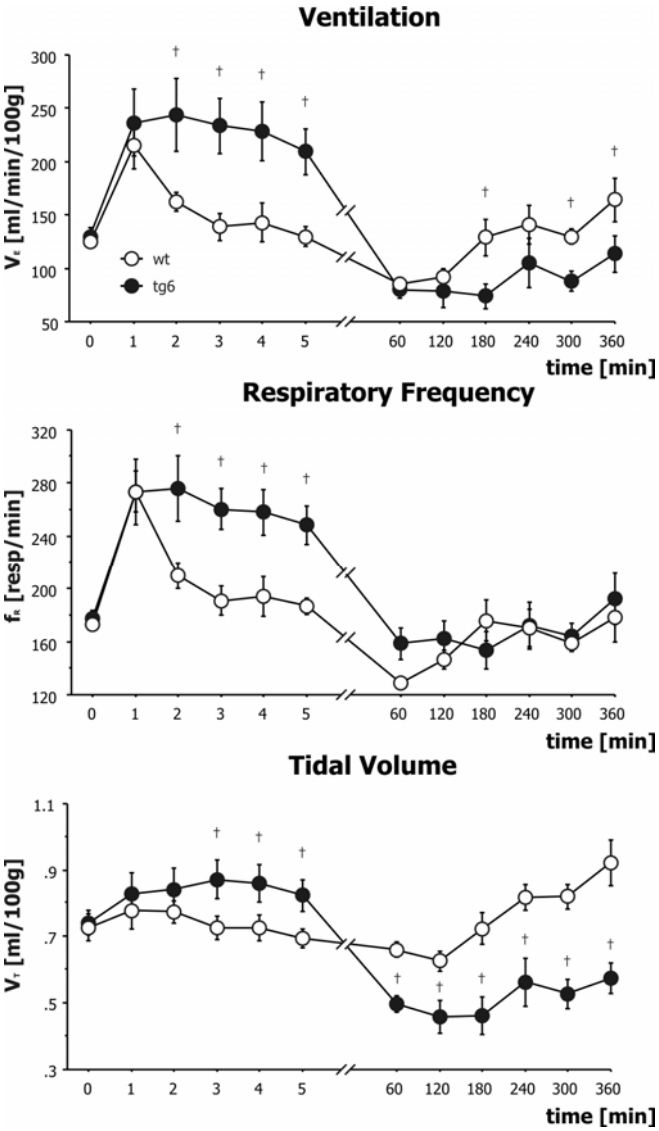



**Figure 3**: Ventilatory response of tg6 and wt mice to 10% inspiratory oxygen concentration for 6 h. For each group n = 6. Data are mean ± SD. * $p < 0.05$ vs corresponding Nx; † $p < 0.05$ vs corresponding wt.

### 5.4 mRNA and protein expression of HIF

#### 5.4.1 HIF-1α and HIF-2α mRNA levels in mouse lung (RT-PCR)

Tg6 and wt mice were exposed to 10% inspiratory oxygen concentration for 24 h, and normoxic animals served as controls. After removing the lung, we measured mRNA expression in this tissue by quantitative real-time PCR. In hypoxia, we observed an increase in HIF-1α mRNA in tg6 and wt mice, but no increase in HIF-2α mRNA. HIF-2α mRNA levels were higher in tg6 than in wt mice under normoxic and hypoxic conditions.

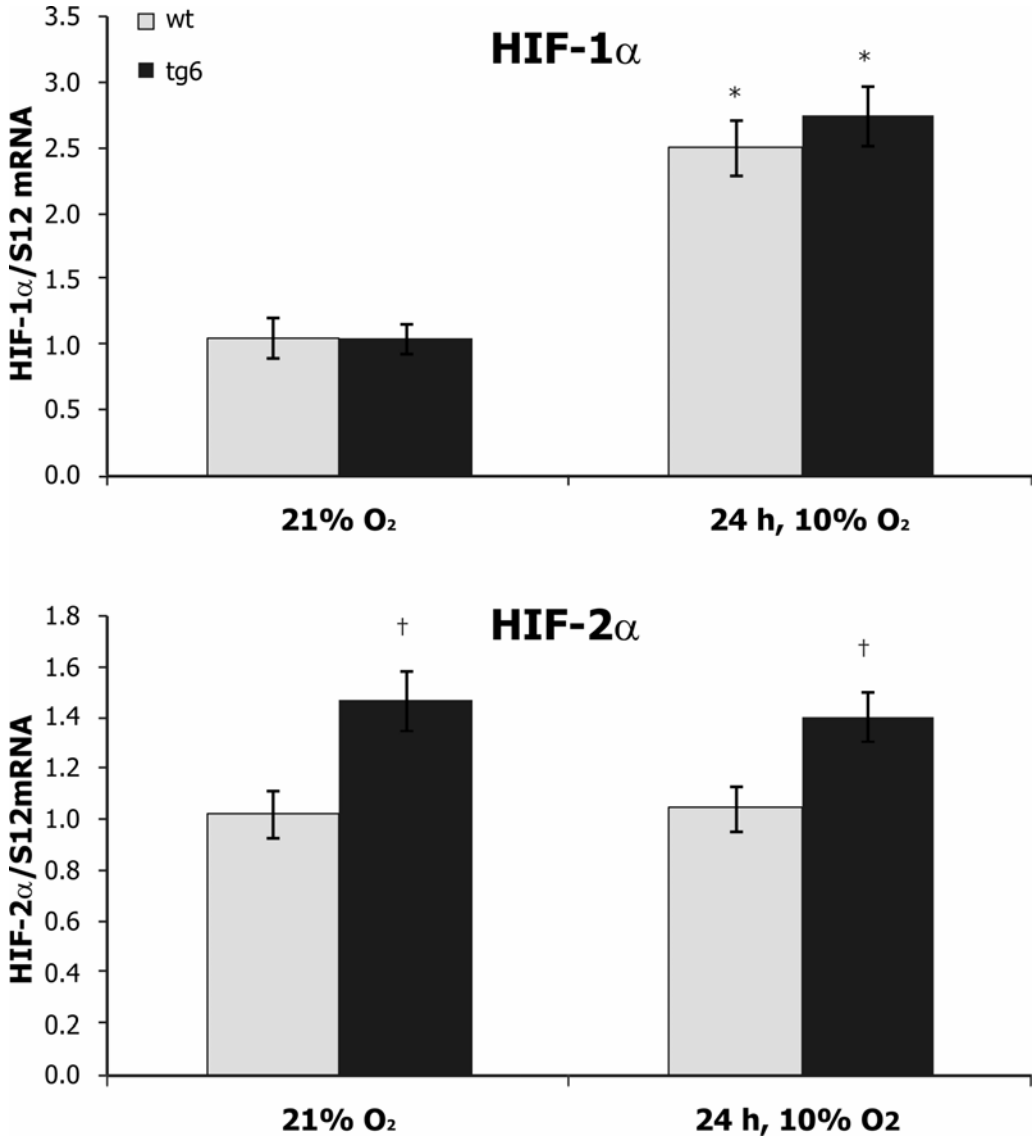



**Figure 4**: HIF1-α and HIF2-α mRNA expression in mouse lungs in normoxia and in response to 10% inspiratory oxygen concentration for 24 h. For each group n = 6. Data are mean ± SD. * $p < 0.05$ vs corresponding Nx; † $p < 0.05$ vs corresponding wt.

### 5.4.2 HIF-1α and HIF-2α protein levels in mouse lung (Western blot)

Knowing that HIF is mainly regulated at the protein level, we analysed HIF stabilisation in hypoxia by measuring HIF protein levels in the lung under normoxic and hypoxic conditions. Tg6 and wt mice were exposed to 12%, 10%, 8% and 7% inspiratory oxygen concentration for 6 h. To analyse the effect of Epo, we injected recombinant human Epo in wt mice prior to hypoxic exposure. In contrast to the mRNA measurements shown above, the protein measurements revealed an increase of HIF-1α (Fig. 5A) and HIF-2α (Fig. 5B) levels under hypoxic conditions in tg6 and wt mice. The increase was more pronounced in tg6 compared to wt animals. As with the haemodynamic measurements, we could not detect a difference in HIF protein levels in wt and Epo treated wt mice.

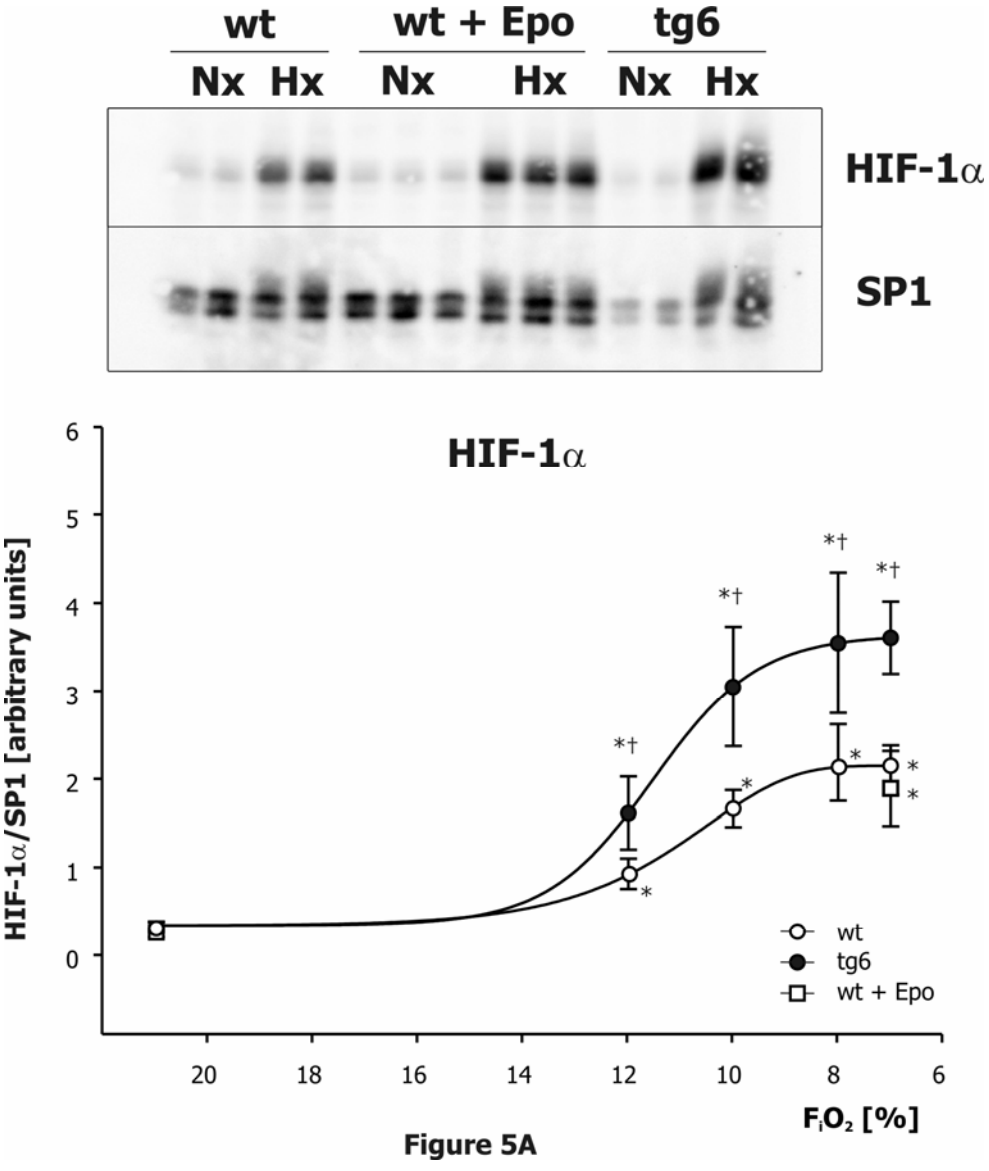



Figure 5A

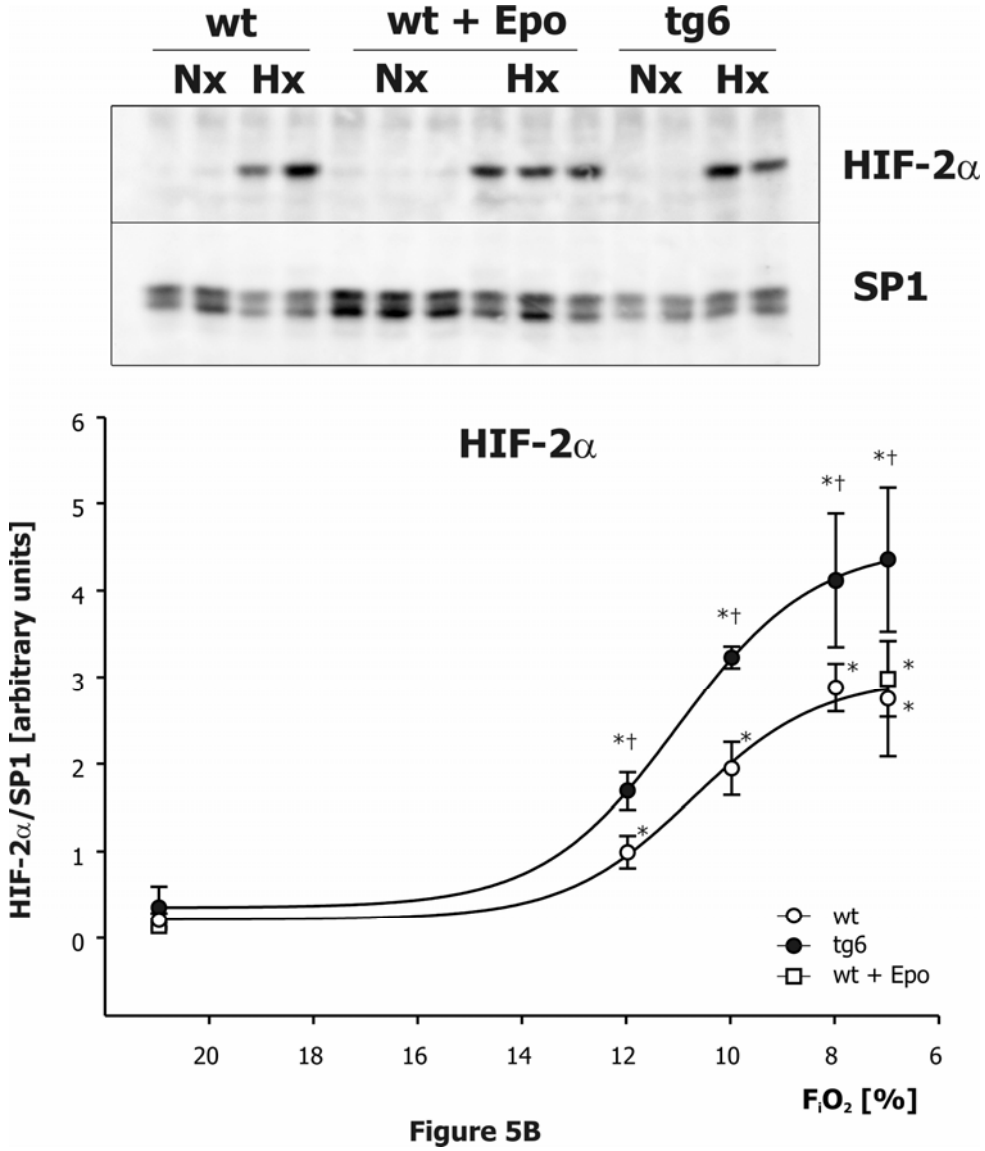



**Figure 5**: Representative Western blots of mouse lungs in normoxia and after 6 h at 7% inspiratory oxygen concentration and dose-response of HIF-1α (Fig. 5A) and HIF-2α (Fig. 5B) accumulation in mouse lung tissue after different inspired oxygen concentrations as indicated for 6 h. The numbers of mice tested are 8, 6, 8, and 12 for wt and 4, 3, 4, and 17 for tg6 at 12%, 10%, 8% and 7% $O_2$, respectively. n = 6 for Epo treated wt mice and n = 3 for normoxic controls in each group. Data are mean ± SD. * $p < 0.05$ vs corresponding Nx; † $p < 0.05$ vs corresponding wt.

### 5.4.3 HIF binding activity in mouse lung (EMSA)

To evaluate whether the enhanced stabilisation of HIF results in an increased HIF activity, we measured HIF binding activity in normoxia and hypoxia by EMSA. Tg6 and wt animals were exposed to 7% inspiratory oxygen concentration for 6 h and again, wt mice were treated with rhEpo prior to the experiment. As a positive control, we used extracts from human Hela cells that produced a fat band slightly above the HIF bands of our mice samples. We observed an increase of HIF binding activity in tg6 and wt mice under hypoxic conditions. The increase in binding activity was higher in tg6 mice compared to wt mice, but there was no difference in wt and Epo treated wt mice.

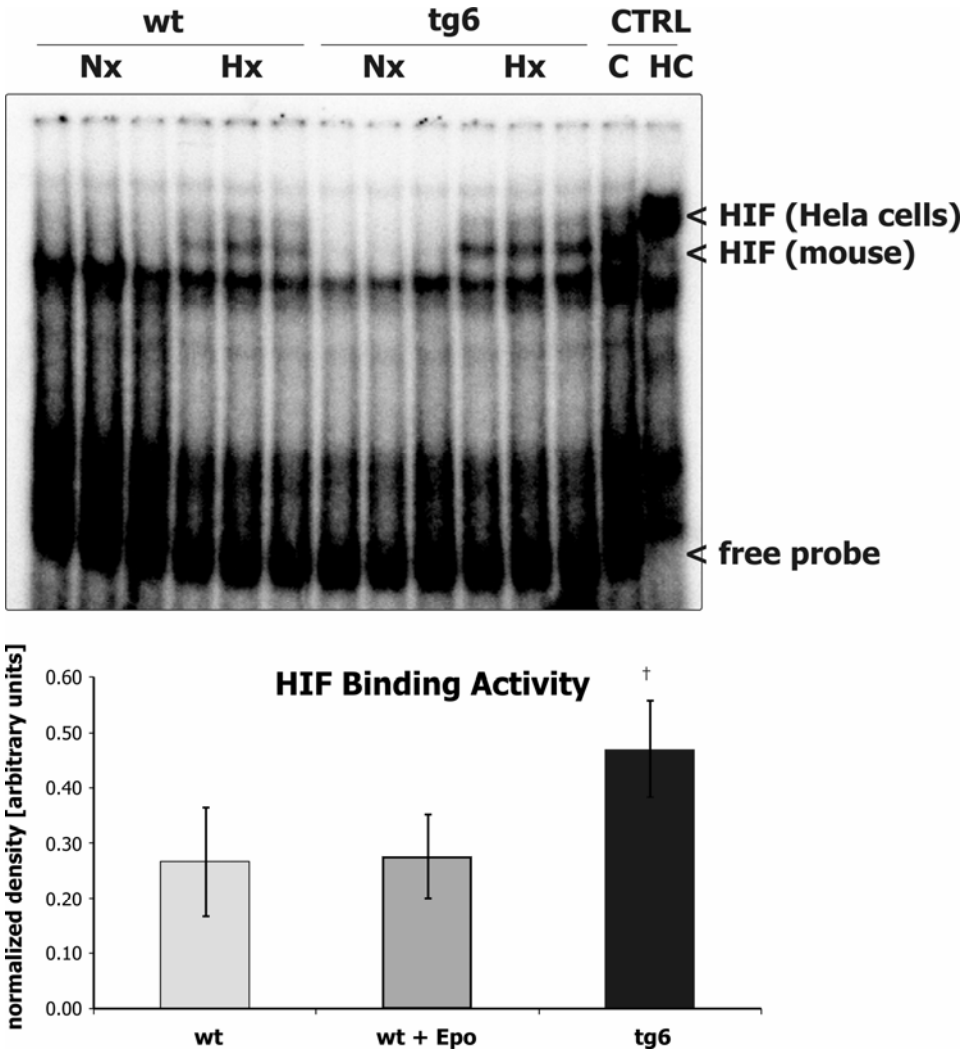



**Figure 6**: Representative EMSA and summary of data of HIF binding activity in mouse lung tissue after inspiratory oxygen concentration of 7% for 6 h. CTRL: control; C: control extracts from a wt mouse (6 h, 6% $O_2$); HC: control extracts from Hela cells (6 h, 1% $O_2$). For each group n = 6. Data are mean ± SD. * $p < 0.05$ vs corresponding Nx; † $p < 0.05$ vs corresponding wt.

### 5.5 PHD1, PHD2 and PHD3 mRNA levels in mouse lung (RT-PCR)

To investigate whether the difference in HIF expression in tg6 and wt mice could be due to different regulation of HIF stabilisation, we measured PHD mRNA levels in normoxia and hypoxia by RT-PCR. Tg6 and wt mice were exposed to normoxia and to 10% or 8% inspiratory oxygen concentration for 24 h. After hypoxia, we observed an increase in PHD3 mRNA in tg6 and wt mice, but not in PHD1 and PHD2. PHD1 and PHD2 mRNA were only slightly increased after severe hypoxia, but only in tg6 mice and not in wt mice. PHD mRNA levels of tg6 and wt mice were comparable in normoxia and hypoxia. Only PHD1 mRNA levels of tg6 mice were slightly higher than the ones in wt mice after severe hypoxia.

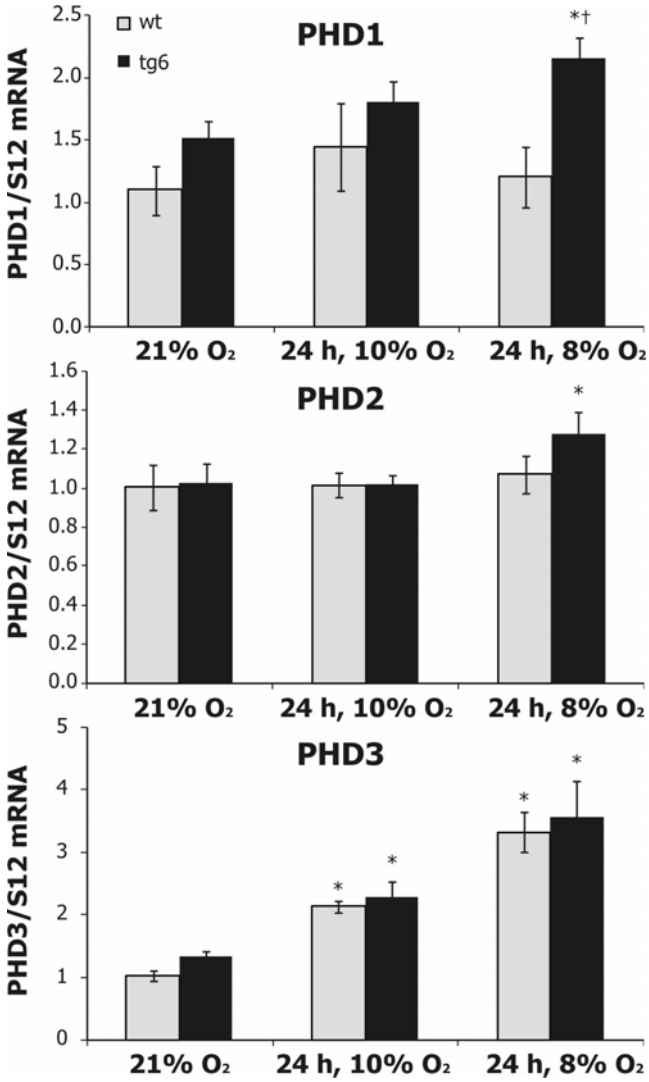



**Figure 7**: PHD1, PHD2 and PHD3 mRNA expression in mouse lungs in normoxia and in response to 10% and 8% inspiratory oxygen concentration for 24 h as indicated. For each group n = 6. Data are mean ± SD. * $p < 0.05$ vs corresponding Nx, † $p < 0.05$ vs corresponding wt.

### 5.6 HIF protein levels upon reoxygenation in mouse lung (Western blot)

Since HIFs are rapidly degraded upon reoxygenation we measured HIF protein levels after different time points of reoxygenation. tg6 and wt mice were exposed to 7% inspiratory oxygen concentration for 6 h and reoxygenated for up to 20 min. We calculated the curve that best fitted the results to define the half life-time (t½) of HIF proteins upon reoxygenation. Assuming an exponential curve, the half-life time of HIF-1α (Fig. 8A) was slightly shorter in tg6 mice relative to wt mice, whereas the half-life time of HIF-2α (Fig. 8B) was the same for both, tg6 and wt mice. Comparing HIF-1α and HIF-2α proteins, we observed only a small difference in the half-life time.

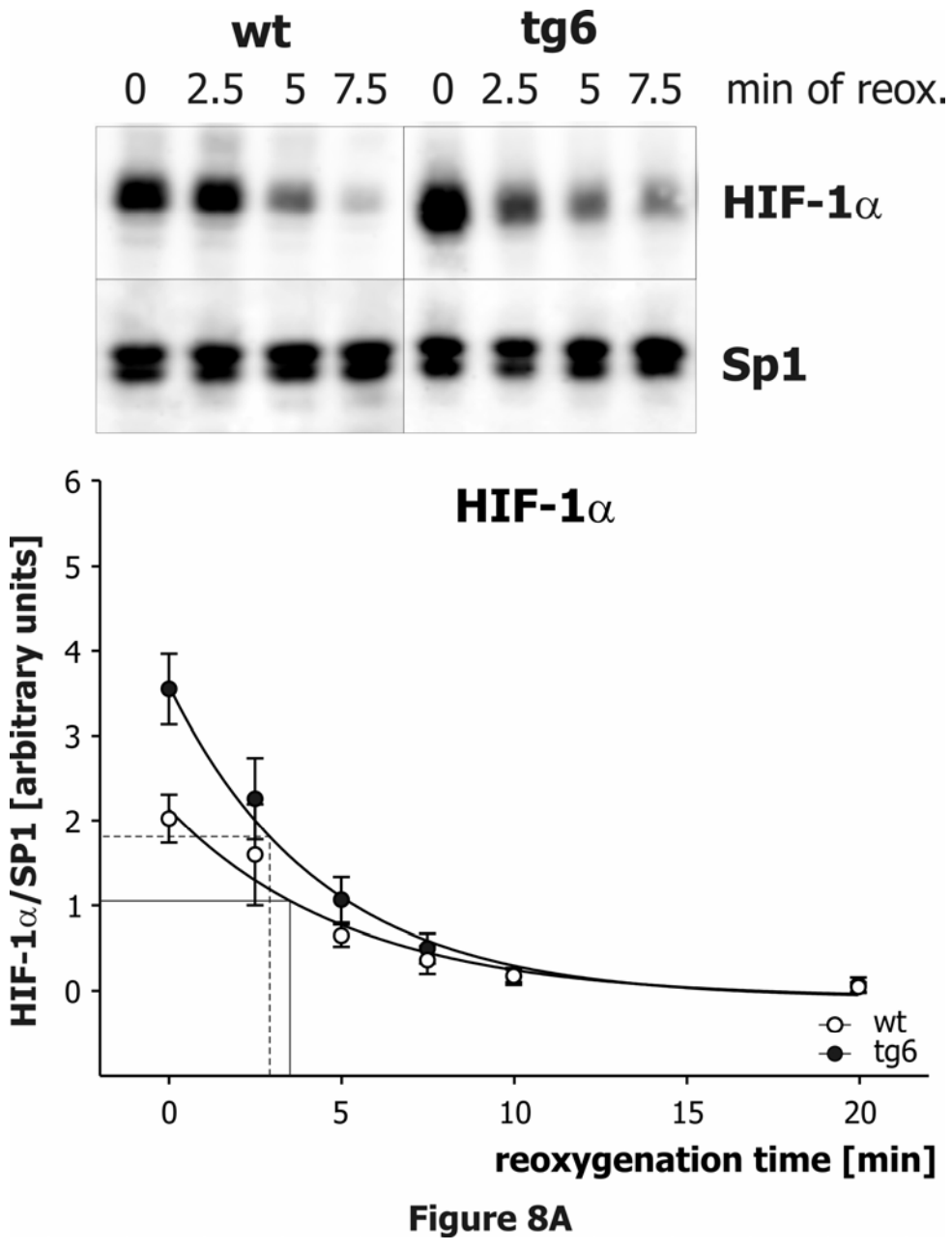



Figure 8A

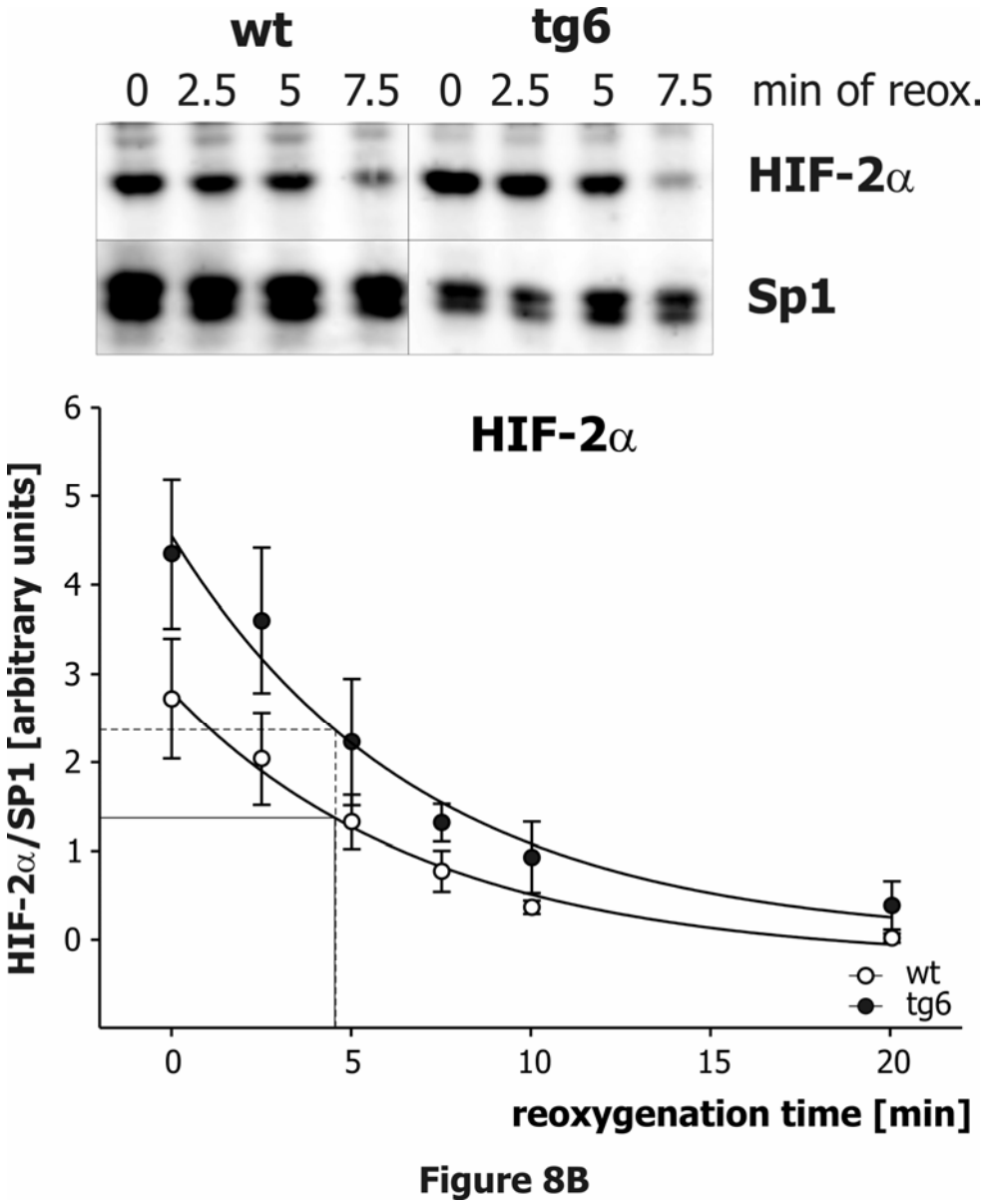



**Figure 8**: Representative Western blots and estimation of HIF-1α (Fig. 8A) and HIF-2α (Fig. 8B) decay rate in mouse lungs upon reoxygenation. Mice were kept at 7% inspiratory oxygen concentration for 6 h. Thereafter, one mouse was sacrificed in the hypoxic workstation (0) and the remaining mice removed to room air and sacrificed after 2.5, 5, 7.5, 10 or 20 min as indicated. For each group n = 6. The half-life times (solid line for wt and dashed line for tg6 mice, respectively) were calculated based on an exponential decay. Data are mean ± SD.

### 5.8 eNOS mRNA in mouse lung (RT-PCR)

Nitric oxide (NO) is one of the strongest vasodilatators known in mammals. Considering that tg6 mice have elevated NO levels in pulmonary endothelial cells, we investigated the influence of hypoxia on the mRNA expression of the endothelial NO synthase (eNOS). We exposed tg6 and wt mice to 10% and 8% inspiratory oxygen concentration for 24 h, and normoxic animals served as controls. There was an increase of eNOS mRNA under hypoxic conditions, but only at an inspired oxygen concentration of 8% but not of 10%, and the increase was higher in tg6 mice compared to wt mice. Tg6 mice also showed higher eNOS mRNA concentrations than wt mice at an inspired oxygen concentration of 10%.

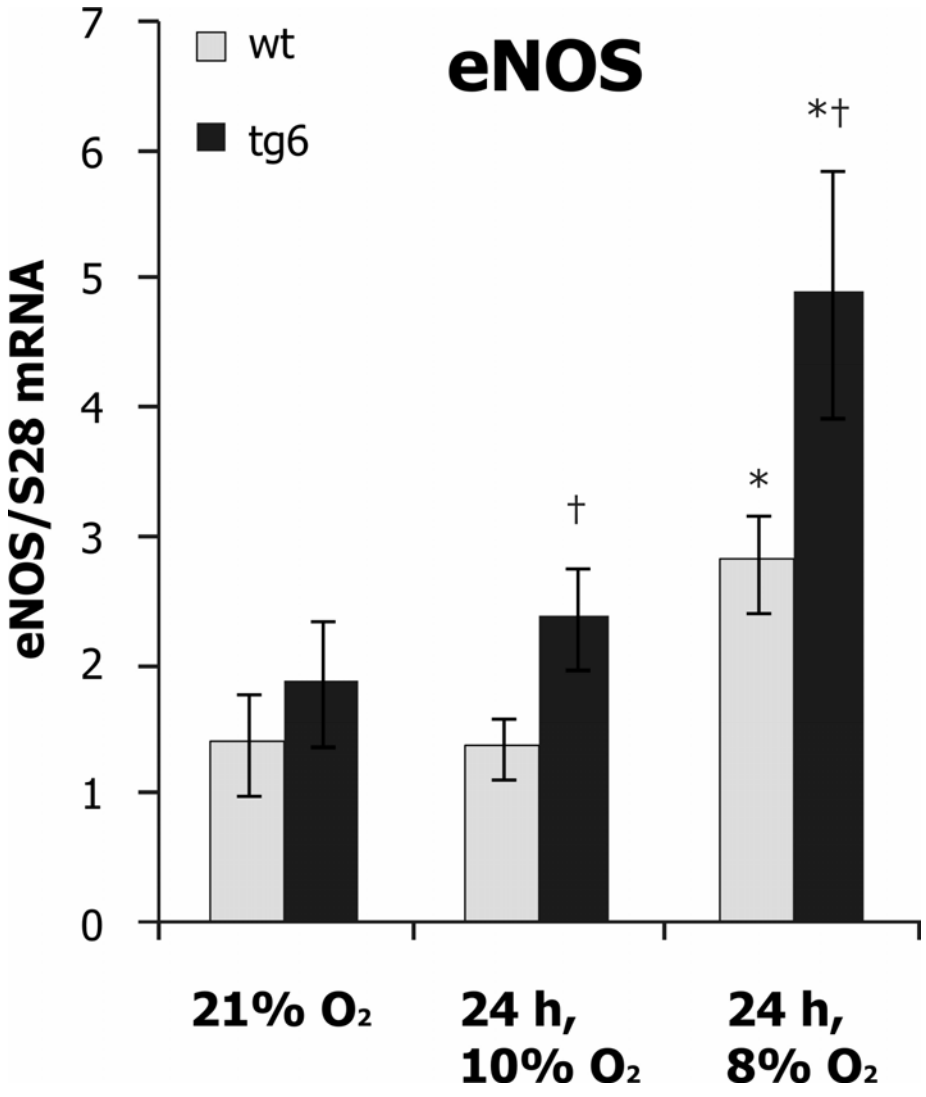



**Figure 9**: mRNA expression of endothelial nitric oxide synthase (eNOS) in mouse lungs in response to normoxia and to 10% or 8% inspiratory oxygen concentration for 24 h. For each group n = 6. Data are mean ± SD. * $p < 0.05$ vs corresponding Nx; † $p < 0.05$ vs corresponding wt.

### 5.7 HIF protein levels in mouse lung after L-NAME treatment (Western blot)

To further investigate the influence of NO on HIF stabilisation, we injected the NO synthase inhibitor L-NAME prior to hypoxic exposure. Before measuring HIF protein levels, tg6 mice were exposed to 10% and wt mice to 8% of inspiratory oxygen for 6 h. We observed a reduction of HIF-1α and HIF-2α protein levels through L-NAME treatment in hypoxia, and the reduction was stronger in tg6 mice compared to wt mice.

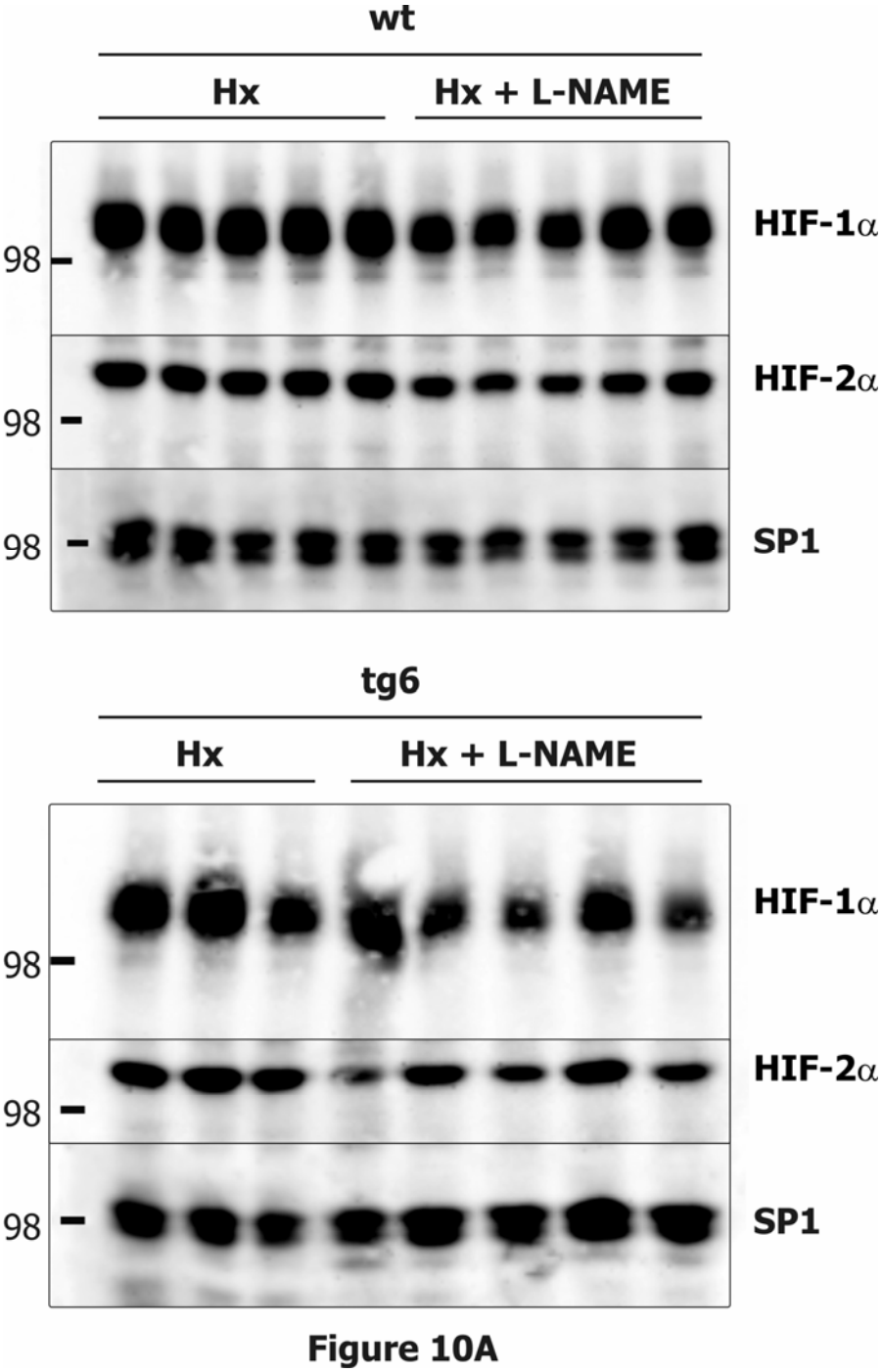



Figure 10A

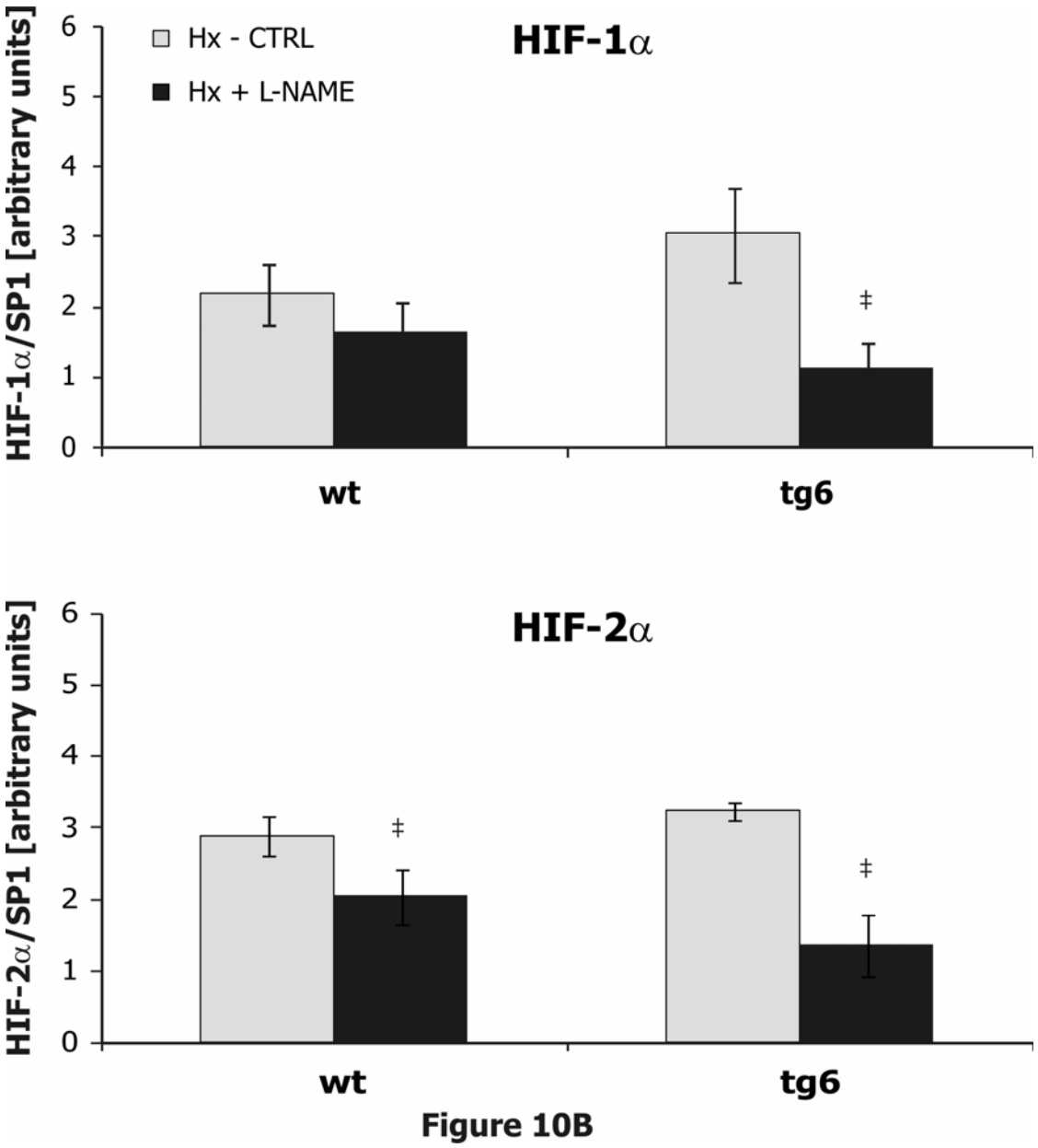



**Figure 10**: Effect of nitric oxide synthase (NOS) inhibition by L-NAME (50mg/kg, i.p.) on HIF-1α and HIF-2α protein accumulation in mouse lungs after inspiratory oxygen concentration of 10% (tg6) or 8% (wt) for 6 h. Representative Western blots (Fig. 10A) and summary of data (Fig. 10B). CTRL: control. Data are mean ± SD. * $p < 0.05$ vs corresponding Nx; † $p < 0.05$ vs corresponding wt; ‡ $p < 0.05$ vs corresponding Hx-CTRL.

## 6. Discussion

The results obtained in the current study indicate that compared to wt mice, tg6 mice are more resistant to developing pulmonary hypertension in response to hypoxia. Interestingly, tg6 mice are at the same time more sensitive to hypoxia, since under hypoxic conditions tg6 mice showed impaired ventilation, reduced blood oxygenation, and elevated HIF levels to a higher extent than the wt mice. The aim of our study was to investigate whether the differences observed in tg6 and wt mice are directly caused by Epo or due to the excessive erythrocytosis developing in tg6 mice.

Since RVSP directly reflects the pressure in the lung, we measured RVSP instead of pulmonary pressure. We evaluated RVSP in tg6 and wt mice under normoxic and hypoxic conditions. Tg6 mice had a higher RVSP and RV $dP/dt_{max}$ compared to wt mice under normoxic conditions. This was not surprising, considering the tremendous increase in haematocrit and blood volume reported in tg6 mice (Ruschitzka 2000; Wagner 2001; Shibata 2003; Vogel 2003), and it is in agreement with previous studies (Deten 2004; Hasegawa 2004).
Both, RVSP and RV $dP/dt_{max}$ dose-dependently increased in wt mice during hypoxic exposure, whereas the tg6 animals did not show a statistically relevant change of either value. Since tg6 mice kept in a normoxic environment have a larger amount of medium-sized and large vessels and a higher percentage of non-muscularised vessels relative to wt mice (Weissmann 2005), the wall tension in their vessels is increased. According to La Place's equation, this could explain their attenuated hypoxic vasoconstriction and smaller increase in RVSP. This is also in line with previous reports that describe altered pulmonary vascular reactivity in tg6 mice (Hasegawa 2004; Weissmann 2005). NO also plays an important role in controlling the vascular tone, and it was shown that NO, among other factors, could attenuate hypoxia-induced pulmonary hypertension (Fagan 1999a; Fagan 1999b). Since the levels of eNOS and NO are higher in the pulmonary arteries of tg6 mice compared to wt mice (Ruschitzka 2000; Hasegawa 2004), a NO-triggered vasodilatation might contribute to the lower pulmonary vasoreactivity of tg6 mice. This could be, at least partly, the explanation why tg6 mice displayed an attenuated haemodynamic response to hypoxia.

The dose-response curves for RVSP and RV $dP/dt_{max}$ of wt mice to decreasing inspired oxygen peaked at an oxygen concentration of 8%. In tg6 mice, we did not observe an increase in RVSP, but we measured the highest values at 12% inspired $O_2$. Furthermore, not all of the tg6 mice survived an inspired oxygen concentration of 6%. This suggested that tg6 mice are more sensitive to hypoxia than wt mice. This observation was surprising, because the decreased vasoreactivity in tg6 mice would rather suggest a delayed maximum of the hypoxic pulmonary response curve. Also, we would expect an increased tolerance of tg6 mice compared to wt animals to low inspiratory oxygen concentrations because of the increased oxygen transport capacity due to the elevated haematocrit levels in tg6 mice.
Since we did not observe a difference in RVSP and RV $dP/dt_{max}$ in wt and Epo treated wt mice, we concluded that Epo most likely does not have a direct effect on RVSP. The altered RVSP occurring in tg6 mice compared to wt mice seemed to be a result of the excessive erythrocytosis in tg6 mice. This was not due to the high blood viscosity though, as previous studies performed in isolated buffer perfused lungs from tg6 mice also showed an attenuated hypoxic vasoconstriction (Weissmann 2005).

The blood gas analysis revealed a lower arterial $pO_2$ and $SO_2$ in tg6 relative to wt mice, but a higher $pCO_2$. This indicates that tg6 mice have an impaired blood oxygenation that could be due to deteriorated gas exchange, reduced ventilation or both. The ventilation analysis showed that tg6 and wt mice displayed an increased breathing rate and consequently elevated minute ventilation after acute exposure to hypoxia. After a short adaptation period, both values returned to normoxic values in wt mice. In contrast, minute ventilation of tg6 animals dropped below the one of wt controls, probably because of a decrease in tidal volume after prolonged hypoxia. These results suggested that tg6 mice have reduced alveolar ventilation under hypoxic conditions, an observation that is in line with previous reports showing altered ventilatory pattern in tg6 mice (Soliz 2007b). Further studies are required to clarify whether this is due to the increased blood volume and erythrocytosis in tg6 mice or if it is a direct effect of Epo, since Epo itself has been shown to modulate the ventilatory response to reduced oxygen (Soliz 2005; Soliz 2007a; Soliz 2007b). The impaired blood oxygenation and ventilation in tg6 mice could explain the decreased tolerance of tg6 mice to low inspired oxygen concentration seen in the haemodynamic measurements.

To investigate at the molecular basis whether the reduced blood oxygenation also leads to tissue hypoxia, we examined the HIF signalling pathway.
Measurements of HIF-1α and HIF-2α mRNA revealed an increase of HIF-1α mRNA during hypoxic exposure for both tg6 and wt mice, whereas HIF-2α mRNA did not change in response to hypoxia. This was in agreement with other studies (Wiener 1996; Palmer 1998). Whereas the HIF-1α mRNA levels in tg6 and wt mice were similar in normoxia and hypoxia, HIF-2α mRNA levels were increased in tg6 animals compared to wt controls.
Assuming that decreased blood oxygenation under hypoxic conditions leads to tissue hypoxia we expected an increase in HIF protein in the tissue during hypoxic exposure. Indeed, protein measurements revealed an increase in both, HIF-1α and HIF-2α protein levels. Furthermore, the increase was more pronounced in tg6 compared to wt mice for both HIF-1α and HIF-2α. This could be explained by the lower arterial $pO_2$ in tg6 mice in hypoxia, which probably leads to a more severe tissue hypoxia. The analysis of the binding activity of HIF by EMSA showed an increase with hypoxia, too. The increase in binding activity was more pronounced in tg6 mice compared to wt mice, as it was for the HIF protein levels. We therefore not only showed that hypoxia leads to HIF protein stabilisation in the lungs of both tg6 and wt mice, but also that the HIF complex was active. Neither protein amount nor binding activity of wt mice and Epo treated wt mice were different. Therefore, we concluded that high Epo plasma levels do not directly influence the regulation of HIF stabilisation. Most probably, secondary effects of Epo are responsible for the differences in tg6 and wt mice.
In contrast to our measurements in the lung, brain tissue from tg6 mice shows lower levels of HIF-1α and HIF-2α in hypoxia than tissue from wt mice (Ogunshola 2006). This could be due to the fact that tg6 mice have a higher transport capacity of oxygen. One explanation for the discrepancy between lung and other tissues could be that under hypoxic conditions, oxygen is redirected to vital organs such as the brain to optimise their oxygenation. HIF levels in the lung could reflect the reduced alveolar oxygen content, which would mean that alveolar epithelial cells are the major source of the observed HIF protein in the tissue. Unfortunately, an identification of the HIF positive cells in the lung by immunohistochemistry was not successful.

To further investigate the differences in HIF stabilisation between tg6 and wt mice, we analysed the PHDs, which are known to be the master regulators of HIF and the most important cellular oxygen sensing system leading to HIF stabilisation (Fandrey 2006). We measured the mRNA levels of PHD1, PHD2 and PHD3. The mRNA levels of PHD3 were increased in hypoxia for both tg6 and wt mice. This was not surprising, because PHD3 and PHD2 are identified as HIF-1α target genes and therefore their expression is induced in hypoxia (Metzen 2003; Fandrey 2006). In our experiment, PHD2 mRNA levels were elevated only after severe hypoxia and only in tg6 mice, but not in wt mice. Unexpected was the small increase of PHD1 mRNA levels after severe hypoxia in tg6 mice, because according to the literature PHD1 is not influenced by hypoxia (Fandrey 2006). Thus, apart from our PHD1 findings, we did not detect any differences in PHD mRNA levels of tg6 and wt mice in normoxia and hypoxia. HIF regulation through PHDs therefore did not explain the difference in HIF stabilisation in tg6 and wt animals. Due to technical problems, we were not able to measure the protein concentrations of PHDs in mouse lung tissue, so instead we analysed the activity of the PHD proteins.
PHD activity is regulated by a number of stimulators and inhibitors including numerous metabolites, reactive oxygen species (ROS) and NO (Kaelin 2008). To circumvent technical difficulties measuring ROS or NO we investigated the capacity of the PHDs to hydroxylate HIF through the measurement of HIF-1α and HIF-2α decay rate upon reoxygenation. Considering the short half-life time of HIF upon reoxygenation (Huang 1996) and assuming that PHDs are the only factors regulating HIF stabilisation, this was a straightforward method for approximating PHD activity. The analysis of the data suggested an exponential decay of HIF upon reoxygenation and a nearly identical half-life for HIF-1α and HIF-2α proteins in tg6 and wt mice, not indicating a reduced PHD activity in tg6 lungs compared to wt lungs. The half-life time of HIF-1α was only slightly shorter in tg6 mice relative to wt mice, which could be due to the higher PHD1 transcript levels in tg6 mice in severe hypoxia. Comparing HIF-1α and HIF-2α, HIF-1α showed a somewhat shorter half-life time than HIF-2α pointing to the possibility that HIF-1α is hydroxylated faster by PHDs than HIF-2α.

Apart from regulating the PHD activity, HIF signalling might be modified by an elevated NO production. Indeed it has been shown that tg6 mice have elevated eNOS and NO levels in the pulmonary artery endothelial cells compared to wt mice

(Ruschitzka 2000; Hasegawa 2004). The assumption that NO influences HIF signalling is supported by our finding that eNOS mRNA levels were augmented with severe hypoxia, and this was more pronounced in tg6 compared to wt animals. We did not observe an induction of eNOS mRNA in moderate hypoxia, but eNOS mRNA was still higher in tg6 compared to wt animals. To evaluate the effect of inhibiting NO synthase, we injected the mice with the NOS- inhibitor L-NAME. This manipulation reduced the protein levels of HIF-1α and HIF-2α under hypoxic conditions in tg6 mice to a much higher extent than in wt mice. The difference in HIF stabilisation in tg6 and wt mice therefore seemed to be at least partly due to changes in NO availability in tg6 mice. Others have reported that Epo might directly activate eNOS (Yamane 1999, Banarjee 2000), but we did not find evidence that Epo directly influences HIF stabilisation, since we could not see a difference in wt and Epo treated wt mice.

In summary, we concluded that upon hypoxic exposure tg6 mice have impaired ventilation with a reduced tidal volume relative to wt animals. Tg6 mice therefore have a more pronounced decrease in alveolar and arterial pO2 followed by an impaired blood and tissue oxygenation. This leads to an increased activation of the HIF signalling pathway. The enhanced accumulation and activation of HIF in tg6 mice seem to be specific for the pulmonary system and linked to the elevated eNOS expression and the resulting enhanced NO production in the tg6 lungs. These findings suggest a higher sensitivity of tg6 mice to hypoxia and can not be explained by systemic elevation of the Epo titer, but are caused by secondary compensatory mechanisms to the excessive erythrocytosis following elevated Epo levels in tg6 animals. The finding that Epo does not directly cause the differences observed in tg6 and wt mice was also documented earlier. It was reported that the infertility of female tg6 mice was not caused directly by Epo but is a result of excessive erythrocytosis (Gassmann 2008). Interestingly, patients with polycythemia vera that causes excessive erythrocytosis without increased Epo plasma levels do not regularly develop pulmonary hypertension. Pulmonary hypertension may be the consequence of local thrombosis in the pulmonary vasculature (Nand 1994; Hachulla 2000), because a prothrombotic tendency due to the high haematocrit and the elevated platelet counts is seen in about 27% of the patients (Dingli 2001). These observations suggest that other factors than excessive erythrocytosis and its consequences are involved in the development of pulmonary hypertension.

To further analyse the effects of Epo, I suggest reducing the haematocrit but not the Epo levels in tg6 mice by splenectomy. Since tg6 mice show extramedullary erythropoiesis in the spleen, splenectomy reduces the haematocrit from 0.89 to 0.62 (Vogel 2003). The resulting mice would show adaptations to excessive erythrocytosis, but would not have elevated haematocrit values despite the increased plasma Epo levels. This would allow distinguishing between direct effects of Epo and secondary effects due to different adapting mechanisms to excessive erythrocytosis in tg6 mice. Increasing the haematocrit but not the Epo levels in wt mice by blood transfusion would also be an exciting experiment.
It would also be interesting to further investigate the organ-specific differences in HIF stabilisation. Besides measuring HIF protein concentrations under normoxic and hypoxic conditions in different tissues, it would be important to analyse tissue NO concentrations, because NO seems to influence HIF stabilisation.

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